5. DISCUSSION

Important banana and plantain varieties are attacked by pathogens of which the most serious is *Fusarium oxysum* f. sp. *cubense* (Stover and Buddenhagen, 1986). The pathogen exists in the soil as chlamydospores (Price, 1982). As plant roots ramify through the soil, root cap cells are sloughed off and root exudates leach out of these cells and the resting spores are able to detect the presence of these nutrients and respond by rapid germination, growth toward the source, and colonization of the root rhizosphere, rhizoplane, and to varying degrees, cells of the epidermis pathogen becomes, cortex and, sometimes, vascular tissues (Pegg, 1989). As the established in the host *F. oxysporum* f. sp. *cubense* spreads into the rhizome stele and then invades the tracheary elements of the pseudostem. Extensive colonization of rhizome xylem occurs prior to invasion of the pseudostem (MacHardy and Beckman, 1981). Conidia are readily taken up and carried in the transpiration stream to sites of entrapment at vessel endings (Beckman, 1990). The microconidia encounter an end wall or perforation plate that permits sap flow but screens out the microconidia (MacHardy and Beckman, 1981). Since the pathogen is obtaining all essential nutrients for normal metabolism from the xylem fluid (Pegg, 1989), the microconidia germinate, and the developing hyphae penetrate the porous barriers and sporulate above the obstruction (MacHardy and Beckman, 1981). Host reaction to the attack by *F. oxysporum* f. sp. *cubense* takes place at this stage. In invaded host plants when the root temperature is above or below the temperatures that are most conducive to disease development (27-28 °C) gels and tyloses that contain infection from the vascular tissues are formed. However, when a virulent pathogen is introduced into a susceptible host at the

optimum temperature for disease development the general defense response become disrupted and permits extensive colonization of the host (Beckman, 1990). As a result of internal water stress (MacHardy and Beckman, 1981), the major symptom of wilt develops when the fungus invades the vascular tissues of petiole and leaves resulting chlorosis and necrosis of leaves (DeVay, 1989). Longitudinal sections of the pseudostem will show signs of vascular discoloration, which will be more distinct in the corm tissue (Nik Masdek *et al.* 1999).

It is now generally accepted that the most effective means of controlling Fusarium wilt is by host resistance and all other means have failed (Pegg *et al.*, 1996) but the most important problem exists in finding adequate methods for screening large number of banana plants for tolerance to Fusarium wilt in short time period. Hwang *et al.* (1984) have mass-produced *in vitro* about 13,000 of Cavendish variety in Taiwan and screened for Fusarium wilt tolerance. Their purpose was to obtain somaclonal variants that could be tolerant/resistant to the pathogen and they only obtained 17 plants or 0.12 per cent. They planted the *in vitro* produced plants in a nursery soil heavily infested with diseased tissue. After 3-4 months, depending on seasonal temperature, the surviving plants are dug up and the rhizomes examined for infection. Those free of infection are again multiplied *in vitro* for additional tests. The process was long and complicated and the need of manpower for such a huge number of plants required for screening somaclonal variants was high. Therefore, the need for screening in easier alternative ways is important.

Important problems associated with earlier (field) screening could be generalized in the following points: a) the rate of disease escapes are higher (Stover and

Buddenhagen, 1986), which has resulted from uneven distribution of pathogen in the field, b) the high demand in terms of cost, manpower and space requirements (Pegg *et al.*, 1996), c) lack of strict quarantine control measures to avoid pathogen spread, d) length of the time required for adequate field infection is too long as plants tend to show symptoms only after 4 to 5 months (Morpurgo *et al.*, 1994), and e) many variables, such as environmental conditions, that may affect infection and symptom expression are difficult to control.

Some of those shortcomings are addressed by the 'Double-tray' technique. The method has the following advantages:

1. Containment of pathogen was effected by the use of the snugly-fitted outer tray.
2. Easy sterilization of contaminated materials where soil is autoclaved and trays could be washed with a solution of sodium hypochlorite (chlorox).
3. The experimental set-up including soil, trays and seedlings is portable and it is thus possible to conduct experiments under pre-determined environmental conditions.
4. The time required for disease expression had been greatly reduced from 4-5 months for field evaluation to between 10 days to 4 weeks.
5. Small space requirement as a single set of trays that can accommodate 20 plantlets per month occupy a bench space of 46×31 cm^2 only.

6. Has ensured screening results that are in agreement with those found in the field screening as 'Intan' nad 'Novaria' remained susceptible and 'Mutiara' and 'Gold Finger' remained tolerant.

The perforated inner tray of the 'Double-tray' technique allowed excess moisture to drain into the outer tray. This has limited the growth of other organisms such as algae on the soil. Plantlets were found to grow well in the set up when water was supplied in alternate day and nutrient solution once a week.

5.1. TECHNIQUE VERIFICATION

5.1.1. Stage of plantlets suitable for screening in the 'Double-tray'

Environmental conditions during hardening phase of tissue cultured plantlets would affect their physiological stage. Some plantlets have more leaves and roots than others and this will have effect on the amount of photosynthates as well as nutrient uptake. Thus, during hardening stage we found that some plants were growing more rapid. The use of plantlets having different physiological stage will have effect on the experimental results, as non-uniform disease expression is likely to occur. We have tried to eliminate all possible problems that could come from the environmental conditions. Plantlets should be hardened under the same moisture, light and temperature conditions and those having same number of leaves, roots and height should be selected. The use of different types of soil in different trays should be avoided.

In the verification experiment, it was observed that seedlings having less than 10 cm tended not to express wilt symptoms and clear external symptoms were expressed by plantlets of 10-15 cm in height. The possible reason for this is that the vascular tissues of plantlets less than 10 cm has not yet fully developed and, as reported by Beckman (1990), adequate establishment of the pathogen in the host is achieved as conidia are taken through the transpiration stream in the vascular tissues. Another possibility is that certain enzymes that are part of the defense mechanism of the plant are more active during early stages of plant growth.

Although Pegg *et al.* (1996) indicated that inoculations of young seedlings at nursery stage could produce severe symptoms that are not expressed in the field, we found that inoculated plantlets of tolerant cultivars in the field were also found to be tolerant when tested using the 'Double-tray' technique. Those plantlets that had survived in the 'Double-tray' challenge, especially 'Mutiara' seedlings, were transferred to the "Fusarium hot-spot" infested with race 4 of the pathogen. The tolerance was still durable after one year in the field.

5.1.2. Symptom expression and spore concentrations

It is important to make considerations on complications that could be involved in screening if the inoculation procedures are not true in nature. Tolerant plants can cope only doses that can be found in the environment; therefore, the challenge must be realistic. Stover and Buddenhagen (1986) indicated that the degree of systemicity and blocking of the vessels is the key to resistance/susceptibilty evaluation; challenge should not be so severe that it precludes systemicity-blocking reactions. In our study, we have tried to select an appropriate inoculum level by examining the trend of disease expression when plantlets are challenged with increasing spore concentrations from low to high. As a result, it has been found that 10^2 spores/ml is too low from the required inoculum dose while 10^6 spores/ml is too high and the realistic spore concentrations are around 10^4 spores/ml.

The results indicate that there was gradual increase in disease severity from healthy at the 10 spores/ml through mild expression at 10^2 spores/ml to high severity

at higher concentrations. Both 'Intan' and 'Novaria' plantlets did not respond to the presence of pathogen when the inoculum level was kept at about 10 spores/ml and the expression has slightly increased at the level of 10^2 spores/ml. More than half of the 'Intan' plantlets did not respond to the presence of the pathogen at 10^2 spores/ml. When the inoculum level was increased to 10^3 spores/ml or more most of the seedlings of the susceptible cultivars produced severe wilt symptoms. The DSI of 'Intan' with respect to different concentrations (Table 4.2) increased from "tolerant" of low concentration, through "susceptible" of medium concentration, to "highly susceptible" of high concentration. The scale indicators of 'Novaria' were similar to that of 'Intan'. This gradual increase of disease expression with increasing inoculum level suggests that the defense mechanism of the plant may work when the pathogen density on the root surface is low. However, if the attack of mycelia are overwhelming and taking place in many parts of the root system, the defense mechanism can no longer be effective. Therefore, the medium level inoculum concentration is middle ground, which could be nearer to the nature.

5.1.3. Duration of root immersion

The duration of root immersion is as important as the inoculum dose and it must be realistic and nearer to what can be found in nature. If plantlets are challenged with inoculum suspension for long periods of time, then the defense mechanisms in tolerant plants could no longer be effective. This is probably what occurred when Vakili (1965) cut roots of young seedlings of *Musa balbisiana* and immersed them in highly concentrated spore suspensions overnight. The plants became systematically

invaded through spore uptake for long time, and their normal field reaction of resistance, exerted through limiting systemicity from few invasion sites, could not be expressed.

In order to find an appropriate duration for inoculation, roots of plantlets were immersed in 10^6 spores/ml inoculum suspension for 2, 2 ½, 3 and 3 ½ hours. All durations produced clear wilt symptoms and the 2-hour duration was recommended to be adopted. This time is long enough to allow mycelia of the pathogen grow inside host cells to ensure a clear disease expression and eliminate the possibility of disease escape, while minimizing the risk of growing plantlets in high density of inoculum level. Our study did not examine a duration shorter than 2 hours but there is a possibility that normal disease expression could be achieved using lesser periods of time for immersion of roots in the suspension, therefore, with this regard, further evaluations are required.

5.1.4. Indices for symptom expression

Since *Fusarium oxysporum* f. sp. *cubense* is soil-borne pathogen, vascular tissues of roots are the first place to be attacked and the pathogen is carried through the transpiration stream. In resistant cultivars colonization is restricted to the region of initial uptake by occlution of infected vessels with gels and tyloses and their lignification at the host-fungal interface (Beckman, 1990). It is, therefore, highly probable that the plant could express presence of the pathogen by discoloration of the occluded vascular elements without showing leaf chlorosis as it is the result of

internal water stress caused by successful establishment of the pathogen inside vascular tissues of the host plant.

Our findings indicate that the rhizome discoloration is the first and most sensitive expression as some tolerant plantlets, such as 'Gold Finger' have shown limited (less than 10%) rhizome discoloration without producing leaf chlorosis (Table 4.11). Even in some inoculated plantlets of the susceptible cultivars, which did not show leaf symptoms showed vascular discoloration (Table 4.9-a, b and c). However, when the level of infection was severe in susceptible cultivars, there was direct relationship between RDI and LSI. The scale levels of 'Novaria' and 'Intan' listed in Table 4.6 indicate that when the level of the LSI increased as a result of increased inoculum density the RDI moved up with it. This suggests that the chlorosis developed in leaves of susceptible plantlets is directly proportional to the discoloration in the rhizome.

5.2. APPLICATIONS OF THE 'DOUBLE-TRAY' TECHNIQUE FOR SCREENING FUSARIUM WILT TOLERANCE

In this study, we have applied the 'Double-tray' technique in examining whether the tolerance/susceptibility of banana cultivars in the field could be reproduced. The technique was also applied in search of somaclonal variants as well as the examining the durability of tolerance in the Taiwanese 'Giant Cavendish' GCTCV-215 which is found to be tolerant to the Taiwanese race 4.

5.2.1. Varietal response to Fusarium wilt pathogen

Although 'Intan' was tolerant at the concentration of 10^2 spores/ml, its status at the concentrations of 10^4 spores/ml and 10^6 spores/ml were susceptible and highly susceptible, respectively. For 'Novaria', 13 of the 30 seedlings inoculated with the low spore concentration of the pathogen did not produce leaf symptoms. However, 10 of them showed varying degrees of rhizome discoloration. The degree of leaf symptom expression and rhizome discoloration intensified with increasing spore concentrations and only one plantlet, which could be a somaclonal mutant, did not show both leaf symptoms and rhizome discoloration at the concentration level of 10^6. These results indicate that 'Intan' has good tolerance for race 4 at lower doses (10^2 or less) of the pathogen while 'Novaria' is susceptible at all concentrations. Apparently the pathogen is proliferating and the inoculum concentration will reach a limit that the

susceptible cultivar can cope but further experiments on the sustainability of this weak tolerance for longer periods of time are required.

The response of the two patches of 'Mutiara' and 'Gold Finger' were different from those of 'Intan' and 'Novaria'. 'Mutiara' variety was the best in terms of showing internal and external symptoms as only five out of the 90 inoculated plantlets had produced symptoms on older leaves by the 3rd week. Even though 'Gold Finger' showed good tolerance at the three spore concentrations, about 50% of inoculated plants produced slight rhizome discoloration, which was limited to small areas at root junctions.

Although the number of plantlets in the tolerant varieties that have demonstrated wilt symptoms was small, the non-uniform exhibition of symptoms is probably the result of one of several factors. It has been attributed to differences in genetic constitution (Vakili, 1965) as well as somaclonal variations that might have developed during tissue culturing (Hwang, 1991). Row and Rosales (1993) cited by Ortiz (1995) indicated that the resistance of bananas to race 4 of *F. oxysporum* f. sp. *cubense* is polygenic while Vakili (1965) mentioned that in the case of race 1 the resistance was conditioned by a single dominant factor. It is also well known that the expression of resistance with systematic invasion in bananas is especially vulnerable to modifications affecting defense mechanisms (Stover and Buddenhagen, 1986). Stress caused by environmental conditions in the greenhouse, such as temperature and light, on physiologically weak plants growing in such confined area with the pathogen could weaken the defense mechanism.

Up to now we know that the results of plantlets inoculated and grown in the 'Double-trays' for 5 weeks are in agreement with those in the field and the 5% of field tolerant cultivars that have shown wilt symptoms could be an indicator that this technique is only valuable for 5 weeks and if the duration becomes more than that the pathogen will proliferate to a limit that will disturb the defense mechanisms of the plantlet. When, for instance, three infected plantlets, which have been showing mild chlorosis on older leaves in the 'Double-trays', were transferred to the hot spot, they recovered within short period of time. Another possibility is that as the pathogen is pulled up and carried in the transpiration stream through the xylem of wounded roots, the amount of damaged roots during uprooting would be more in some plantlets and consequently uptake of the pathogen and severity of symptom expression would be higher. Further experiments on the length of time in which the 'Double-tray' technique is useful before the pathogen is proliferated and under different temperature and light intensities should be conducted. Another suggestion is that to minimize variations in symptom expression all plantlets must be uniform in terms of number of leaves, height and length of roots. Handling of plantlets must be conducted in similar way from one plantlet to the other.

The overall conclusion drawn from these experiments is that, on 'Doublle-tray' technique, the cultivars 'P. Intan" and 'Novaria' are highly susceptible to *F. oxysporum* f. sp. *cubense* race 4 while 'P. Mutiara' and 'Gold Finger' are tolerant and these findings are in agreement with those obtained from field evaluation in the "Fusarium Hot-spot" screening.

5.2.2. Response of GCTCV-215 to the Malaysian isolate of race 4

The results of the GCTCV-215 obtained from the 'Double-tray' technique were different from the tolerance to the Taiwanese race 4 reported by (Hwang, 1991) as the cultivar showed high susceptibility to the Malaysian race 4. Since the earlier results obtained from other cultivars on the 'Double-tray' technique agreed with that of the field evaluation, we attributed this phenomenon to a differential tolerance/susceptibilty by GCTCV-215 to different isolates of race 4 (the Malaysian and the Taiwanese) but the application of the technique has served its purpose. Some authors reported the possibility of pathogen variability from one region to the other (Stover and Buddenhagen, 1986) and as a result pathogenecity and the level of aggressiveness of the two isolates are different. Another possibility is that the cultivar response to the pathogen under different environmental conditions could be variable.

5.2.3. Screening of somaclonal variants

When the 500 plantlets of 'Intan' and the 50 plantlets of 'Novaria were screened the number of variants obtained regarding tolerance to Fusarium wilt were 4 and 2, repectively. The survived 'Novaria' plantlets were transferred to a hot spot infested with *F. oxysporum* f. sp. *cubense* race 4 and after 5 months in the field they are still surviving. Further *in vitro* multiplications on those survived plants and subsequent screenings are required to obtain tolerant variants. The advantage of using 'Double-tray' technique is that the long time required by the other screening methods was shortened and the number of meristem-derived plantlets for screening could be

doubled or tripled, hence, the chances of obtaining more variants could be higher. In conclusion, the somaclonal variants experiment has successfully proven that the 'Double-tray' technique is potentially useful screening method.

APPENDIX

METHODOLOGY OF MEDIA PREPARATION

1. Modified Murashige and Skoog (MS) Medium used for tissue culturing (Dirr, 1987).

Murashige and Skoog medium used for tissue culturing of banana consists of the following ingredients:

KNO_3	1.9 g
$NH4NO_3$	65.0 g
$CuSO_4$	0.025 mg
$MgSO_4$	0.37 g
$MnSO_4$	0.0169 g
$ZnSO_4$	0.0086 g
$CaCl_2 \cdot 2H_2O$	0.44 g
$CoCl \cdot 6H_2O$	0.025 mg
KI	0.83 mg
H_3BO_3	0.0062 g
KH_2PO_4	0.17 g
$NaMoO_4 \cdot 2H_2O$	0.25 mg
$FeSO_4$	0.0287 g

Na_2EDTA	0.0372;
Distilled water	1 L

10 ml of stock solution of vitamins was added to 1 L of MS medium. The stock solution consists of:

glycine	200 mg/L
myo-inositol	10 g/L
nicotinic acid	50 mg/L
pyridoxine HCl	50 mg/L
thiamine HCl	100 mg/L

For shooting, the medium was amended with 1-5 mg BAP/L. For rooting, it was amended with 1-3 mg of IAA/L (Dirr, 1987).

2. Houglands Nutrient solution used for plantlet watering (Epstein, 1972).

The solution was prepared in 1L distilled water and autoclaved at 121 C for 30 minutes. The ingredients were:

KH2PO4	0.136 g
KNO_3	0.5055 g
$Ca(NO_3)_2 \cdot 4H_2O$	1.181 g
$MgSO_4 \cdot 7H_2O$	0.23 g,
KCl	3.728 mg
H_3BO_3	1.546 mg
$MnSO_4 \cdot H_2O$	0.338 mg

$ZnSO_4 \cdot 7H_2O$	0.575 mg
$CuSO_4 \cdot 5H_2O$	0.125 mg
H_2MoO_4 (85% MoO_3)	0.081 mg
Fe-EDTA	0.922 mg

3. **Dehaydrated Potato Dextrose Agar (PDA) used for isolation of Fusarium oxysporum f. sp. cubense race 4 from diseased plant tissues (Singleton *et al.*, 1992).**

The medium was prepared from:

Difco potato dextrose agar	39 g
Distilled water	1 L

The mixture was autoclaved at 121 C for 30 minutes and distributed in Petri dishes.

4. **Water agar used for single spore isolation (Booth, 1977).**

It consists of:

Difco Bacto agar	15-20 g
Distilled water	1 L

The mixture was autoclaved at 121 C for 30 minutes and distributed in Petri dishes.

5. Armstrongs' Liquid Medium used for the preparation of inoculum suspension (Singleton, 1992).

The ingredients of the solution was consisting of:

Sucrose	20 g
$MgSO_4 \cdot 7H_2O$	400 mg
KCl	1.6 g
KH_2PO_4	1.1 g
$Ca(NO_3)_2$	5.9 g
$FeCl_3$	0.2 µg/ml
$MnSO_4$	0.2 µg/ml
$ZnSO_4$	0.2 µg/ml
Distilled water	1 L

The mixture was autoclaved at 121 C for 30 minutes and distributed in 500-ml bottles.